Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 16 (2006) 5132–5135
Bioorganic & Medicinal Chemistry Letters

Protein microarrays to study carbohydrate-recognition events

Myung-ryul Lee, Sungjin Park and Injae Shin*

Department of Chemistry, Yonsei University, Seoul 120-749, Republic of Korea

Received 16 May 2006; revised 4 July 2006; accepted 7 July 2006
Available online 27 July 2006

Abstract—In order to expand areas in which protein microarrays can be used to solve important biological problems, we have investigated ways in which the technique can be employed for functional glycomics. Initially, our protein microarrays were used for the rapid identification of carbohydrate-binding proteins using trifunctional carbohydrate probes and fluorescent dye-labeled polysaccharides. Glycan probes were selectively bound to the corresponding lectins immobilized on the solid surface. In addition, these microarrays were also employed for profiling of carbohydrates on Jurkat T-cell surfaces. These cells adhered to ConA, RCA_{120}, SNA and WGA, indicating expression of α-Man, Gal, NeuNAcα2,6Gal and GlcNAc residues on their surfaces. Furthermore, we determined binding affinities between WGA and carbohydrates by measuring IC_{50} values of GlcNAc that inhibited 50% of trivalent GlcNAc binding to WGA immobilized on the solid surface. All the experiments show that protein microarrays can be used to study carbohydrate-recognition events in the field of glycomics.

Functional studies of proteins revealed in the recently completed genome sequence have been concentrated on elucidating their biological functions and developing novel pharmaceutical agents that interact with proteins involved in disease-related physiological processes. Among technologies developed for this purpose, the protein microarray has received considerable attention and emerged as a powerful tool for biological and biomedical research.[1] This microarray technology has been used to identify binding partners, such as proteins, DNA, and small molecules, by analyzing protein–protein, protein–DNA, and protein–small molecule interactions.[2] In addition, protein microarrays are employed to analyze the biochemical activities of enzymes, such as kinases or hydrolases, and to understand metabolic pathways.[3] These microarrays are also applied for profiling disease markers or autoimmune responses by assessing protein–antibody interactions in biomedical research.[4] Furthermore, protein microarrays have been used for high-throughput screening of inhibitors or activators.[5]

A major driving force behind the expanded use of protein microarrays is the development of new experimental protocols in which they can be applied. A potentially important application of these microarrays is the rapid identification of lectins found in the proteome, since lectins are involved in a variety of physiological and pathogenic process through their interactions with carbohydrates.[6] According to analysis of the human genomic sequence, it is suggested that about 100 gene products are lectins.[7] However, the carbohydrate-binding properties of only about half of these lectins have been relatively well studied; the functions of the remaining proteins in this family have not yet been elucidated. Thus, it is urgent to identify these unknown lectins in order to understand the biological implications of carbohydrate–protein interactions and to help develop new carbohydrate-based pharmaceutical agents. As described below, we have applied protein microarrays for the fast identification of proteins that bind to mono-, di- or polysaccharides, and, importantly, for the detection of proteins that interact with mammalian cells.[8,9] In addition, we have used this microarray technology for the rapid determination of binding affinities between lectins and carbohydrates.

Protein microarrays used in this study were fabricated by printing proteins on *N*-hydroxysuccinimide (NHS)-derivatized glass slides. The epoxide-coated slides were reacted with 4,7,10-trioxa-1,13-tridecanediamine.[8c] The resulting amine-modified slides were treated with succinic anhydride and subsequently NHS in the presence of diisopropylcarbodiimide (DIC) to give NHS-coated slides.

Keywords: Carbohydrates; Carbohydrate–protein interactions; Immobilization; Lectins; Protein microarrays.
* Corresponding author. Tel.: +82 2 21232631; fax: +82 2 3647050; e-mail: injae@yonsei.ac.kr

0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.07.028

Trifunctional probes that possess a carbohydrate ligand, a fluorescent dye (Cy3), and a photoreactive benzophenone group were used to detect lectins on the protein microarrays (Fig. 1). These probes were synthesized by using a solid-phase methodology based on a safety-catch strategy.[10] The fluorescent dye (Cy3) was incorporated into these probes in order to detect bound lectins and the photoreactive benzophenone group was inserted to promote covalent labeling of weakly bound proteins. Since interactions between monovalent carbohydrates and proteins are typically weak, trivalent carbohydrate probes were also prepared to facilitate detection of proteins immobilized on surfaces through cluster effects.[10]

Initial exploratory work was performed to optimize the conditions for immobilization and binding of the trifunctional probes to proteins. It was revealed that protein microarrays irradiated after incubation with the trifunctional probes exhibit stronger fluorescent intensities than those that are not irradiated (Fig. 2). Irradiation for 30 min is ideal for the strong binding of the probes to proteins. However, longer irradiation time (60 min) causes nonspecific interaction of the probe with proteins. In addition, trivalent carbohydrate probes have higher binding affinities than their monovalent counterparts under both irradiation and non-irradiation conditions. Furthermore, the use of 15–20 μM concentrations of proteins is sufficient to enable the efficient binding of carbohydrates and 0.5–1.0 μM concentrations of the probes result in good binding properties.

To demonstrate that protein microarrays, prepared by using the procedure described above, can be used to rapidly identify carbohydrate-binding proteins, 20 proteins

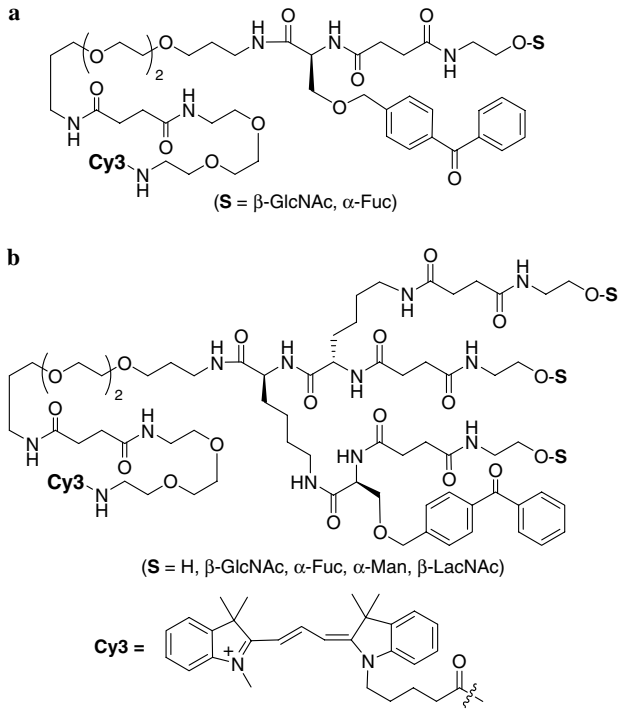

Figure 1. Structures of (a) mono- and (b) trivalent carbohydrate probes for the detection of carbohydrate-binding proteins on protein microarrays.

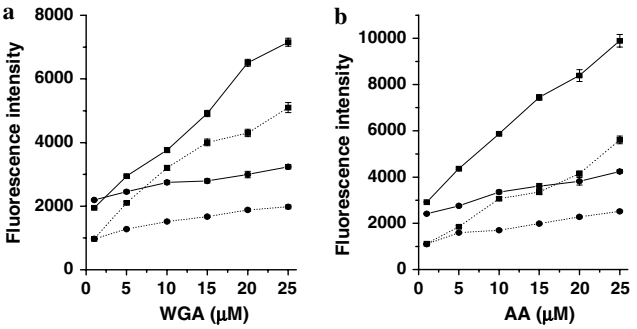

Figure 2. Effects of immobilization concentrations of (a) WGA and (b) AA on binding of Cy3-GlcNAc (0.5 μM) and Cy3-Fuc (0.5 μM) probes, respectively (■, trivalent probes; ●, monovalent probes; solid line, irradiation for 30 min; dot line, non-irradiation).

(15 μM) were printed on NHS-coated slides by using a pin-type microarrayer. The proteins include lectins (*A. aurantia* (AA), ConA, RCA_{120}, *Sambucus nigra* (SNA), and wheat germ agglutinin (WGA)), proteases (collagenase, pepsin, trypsin, and thrombin), glycosidases (amylase, hyaluronase), phosphatases (alkaline phosphatase, RNase A), kinases (myokinase), and others (antithrombin, glutathione-*S*-transferase, insulin, ovalbumin, penicillin G amidase, and streptavidin). After incubation for 4 h at room temperature in a humid chamber, the slides were blocked with BSA for 0.5 h. The resulting slides were treated with trivalent Man, Fuc, GlcNAc, and LacNAc probes (0.5 μM) and then irradiated for 30 min at 4 °C. The results show that ConA, AA, and WGA on the microarray selectively interact with Man, Fuc and GlcNAc, respectively (Fig. 3a–c), while the other proteins do not bind to these carbohydrates.[11] When the microarray was incubated with a LacNAc probe, both RCA_{120} (a Gal-binding protein) and WGA interacted with this probe, which is consistent with the previous results (Fig. 3d).[12] As a control,

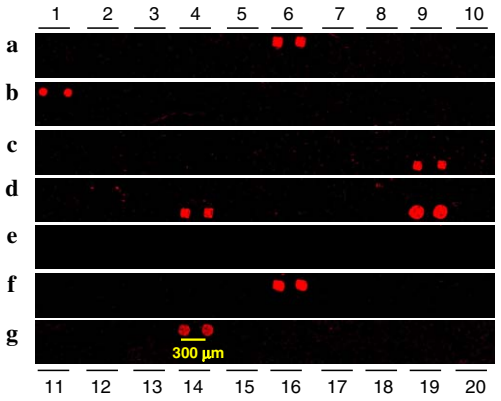

Figure 3. Fluorescence images of protein microarrays containing twenty proteins incubated with (a) trivalent Cy3-Man (0.5 μM); (b) trivalent Cy3-Fuc (0.5 μM); (c) trivalent Cy3-GlcNAc (0.5 μM); (d) trivalent Cy3-LacNAc (0.5 μM); (e) trivalent probe (0.5–5 μM) without a sugar moiety (S = H in Fig. 1b); (f) Cy3-mannan (100 μg/mL); and (g) Cy3-heparin (100 μg/mL). 1, AA; 2, alkaline phosphatase; 3, amylase; 4, antithrombin; 5, collagenase; 6, ConA; 7, glutathione-*S*-transferase; 8, hyaluronase; 9, insulin; 10, myokinase; 11, ovalbumin; 12, pepsin; 13, penicillin-G-amidase; 14, RCA_{120}; 15, RNase A; 16, streptavidin; 17, SNA; 18, trypsin; 19, WGA, and 20, thrombin.

the protein microarray was treated with a trivalent probe (0.5–5.0 μM) without a sugar moiety (S = H in Fig. 1b) and then irradiated for 30 min. This probe did not bind to the lectins, indicating that the carbohydrate moieties in the probes are critical for lectin binding (Fig. 3e).

This technique is also applicable for the identification of polysaccharide-binding proteins. Fluorescent dye-labeled polysaccharides required for this study were obtained by reacting polysaccharides (mannan and heparin) with Cy3–NHS in DMSO–pyridine or H_2O. Probing the protein microarray with Cy3-labeled mannan shows that mannan containing the highly branched α-mannose interacts only with ConA (Fig. 3f). The protein microarrays were also exploited to detect glycosaminoglycan (GAG)-binding proteins. The protein microarray probed with Cy3-labeled heparin shows that this GAG interacts only with antithrombin, an inhibitor of blood coagulation enzymes (Fig. 3g).[13] It is important to note that more than one hundred proteins have been reported to bind GAGs (e.g., hyaluronic acid, heparin/heparin sulfate, chondroitin sulfate, and keratin sulfate).[14] Interactions of these proteins with GAGs are involved in various physiological processes such as homeostasis, cell growth, cell migration, and development. Therefore, the development of protein microarrays for the detection of GAG-binding proteins is a significant finding.

The applicability of the microarray technique to the detection of proteins that interact with mammalian cells was also probed. Jurkat T-cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin, at 37 °C. The cells were collected by centrifugation, suspended in PBS or a culture medium, incubated with SYTO 83 for 30 min for their labeling, and applied to the microarray containing twenty proteins. Alternatively, the suspended cells were poured onto the slide and then, following washing to remove unbound cells, fixed by treatment with 4% formaldehyde for 30 min. The cells bound to the proteins on the slide were stained by 30-min treatment with SYTO 83.

This exploration led to several important conclusions. First, T-cells suspended in PBS bind to lectins more tightly than those suspended in a culture medium. Second, the cells stained after fixation with formaldehyde exhibit stronger fluorescent intensities than those pre-incubated with the dye. It was also reported that pre-incubation of $CD4^+$ human T-cells with a dye affected cell binding to carbohydrates on the carbohydrate arrays.[8g] Third, T-cells adhere to ConA, RCA_{120}, SNA, and WGA but did not bind to other proteins. The binding results indicate that T-cells express α-Man, Gal, NeuNAcα2,6Gal, and GlcNAc residues on their surfaces, which is consistent with the previous results (Fig. 4).[15] In fact, it is known that certain lectins promote the transformation of cells from the resting states to blast-like cells through their interactions with cell surface carbohydrates. This subsequently leads to mitotic division.[16] Therefore, the discovery that an evaluation

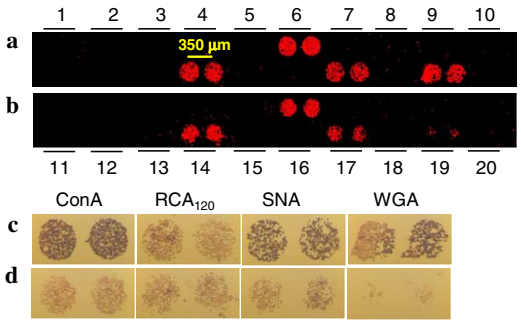

Figure 4. Fluorescence images of protein microarrays (spotting volume; 2 nL) incubated with (a) 10^8 and (b) 10^7 Jurkat T-cells followed by fixation and staining with SYTO 83. Microscopic images of the cells bound to proteins on the microarrays after treatment with (c) 10^8 and (d) 10^7 Jurkat T-cells.

of interactions between potent mitogenic lectins from the proteome and cell surface carbohydrates can be performed by using protein microarrays should serve as an important contribution to the methods available for advancing the understanding of cell growth and development.

Finally, the use of protein microarrays for the rapid determination of the binding affinities between lectins and carbohydrates was investigated. This study was performed by determining the concentration (IC_{50}) of GlcNAc needed to inhibit 50% of trivalent GlcNAc binding to WGA on the microarray under irradiation and non-irradiation conditions. Slides containing microspots of WGA were incubated with mixtures of a trivalent GlcNAc probe (0.5 μM) containing various concentrations (0.1–200 mM) of GlcNAc for 1 h. After washing the slides to remove unbound probes, the amount of a bound probe was quantitated by determining its fluorescent intensity. Alternatively, the incubated slides were irradiated for 30 min and then the fluorescent intensity was determined to analyze the amount of bound probe. The IC_{50} values of GlcNAc that inhibited 50% of trivalent GlcNAc binding to WGA immobilized on the solid surface were determined to be 7.9 and 17.0 mM under non-irradiation and irradiation conditions, respectively (Fig. 5). The concentration of methyl

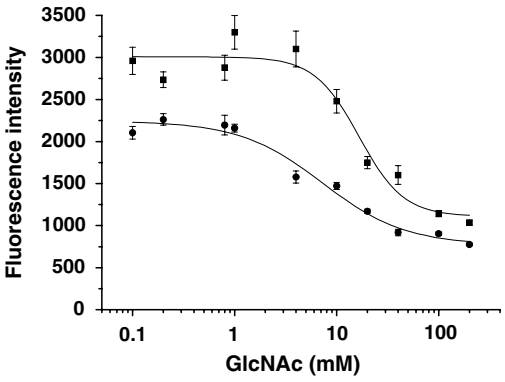

Figure 5. Determination of concentrations (IC_{50}) of GlcNAc to inhibit 50% of trivalent GlcNAc binding to WGA on the protein microarray under irradiation (■) and non-irradiation (●) conditions.

β-GlcNAc to inhibit 50% of hemagglutination activity of WGA was known to be about 10 mM.[17] Thus, quantitative data obtained from protein microarrays are comparable to those measured by other methods.

Protein microarrays prepared by immobilizing proteins on the NHS-coated slides have been applied to the important field of glycomics. The designed trifunctional probes are suitable for detecting carbohydrate-binding proteins on the protein microarrays. Recently, proteome microarrays have been employed for several areas of biological research. The present work suggests that proteome microarrays could serve as a method to simultaneously identify a number of unknown carbohydrate-binding proteins from the proteome. Once unknown lectins are identified in this way, their binding properties can be investigated in a high-throughput manner using carbohydrate microarrays. Thus, a cooperative dual-microarray technology can be envisaged for studying biological processes associated with carbohydrate-protein interactions.

Acknowledgments

This work was supported by grants from the NRL program and the SRC program of MOST/KOSEF (R11-2000-070-04003-0).

Supplementary data

Experimental procedure. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2006.07.028.

References and notes

1. (a) Bertone, P.; Snyder, M. *FEBS J.* **2005**, *272*, 5400; (b) Tomizaki, K. Y.; Usui, K.; Mihara, H. *ChemBioChem* **2005**, *6*, 782; (c) LaBaer, J.; Ramachandran, N. *Curr. Opin. Chem. Biol.* **2005**, *9*, 14; (d) Predki, P. F. *Curr. Opin. Chem. Biol.* **2004**, *8*, 8; (e) Wilson, D. S.; Nock, S. *Angew. Chem., Int. Ed.* **2003**, *42*, 494.
2. (a) Hall, D. A.; Zhu, H.; Zhu, X.; Royce, T.; Gerstein, M.; Snyder, M. *Science* **2004**, *306*, 482; (b) Fang, Y.; Frutos, Y. G.; Lahiri, J. *J. Am. Chem. Soc.* **2002**, *124*, 2394; (c) Zhu, H.; Bilgin, M.; Bangham, R.; Hall, D.; Casamayor, A.; Bertone, P.; Lan, N.; Jansen, R.; Bidlingmaier, S.; Houfek, T.; Mitchell, T.; Miller, P.; Dean, R. A.; Gerstein, M.; Snyder, M. *Science* **2001**, *293*, 2101; (d) MacBeath, G.; Schreiber, S. L. *Science* **2000**, *289*, 1760.
3. (a) Jung, G. Y.; Stephanopoulos, G. *Science* **2004**, *304*, 428; (b) Chen, G. Y. J.; Uttamchandani, M.; Zhu, Q.; Wang, G.; Yao, S. Q. *ChemBioChem* **2003**, *4*, 336; (c) Zhu, H.; Klemic, J. F.; Chang, S.; Bertone, P.; Casamayor, A.; Klemic, K. G.; Smith, D.; Gerstein, M.; Reed, M. A.; Snyder, M. *Nat. Genet.* **2000**, *26*, 283.
4. Robinson, W. H.; Fontoura, P.; Lee, B. J.; de Vegvar, H. E. N.; Tom, J.; Pedotti, R.; DiGennaro, C. D.; Mitchell, D. J.; Fong, D.; Ho, P. P. K.; Ruiz, P. J.; Maverakis, E.; Stevens, D. B.; Bernard, C. C. A.; Martin, R.; Kuchroo, V. K.; van Noort, J. M.; Genain, C. P.; Amor, S.; Olsson, T.; Utz, P. J.; Garren, H.; Steinman, L. *Nat. Biotechnol.* **2003**, *21*, 1033.
5. (a) Funeriu, D. P.; Eppinger, J.; Denizot, L.; Miyake, M.; Miyake, J. *Nat. Biotechnol.* **2005**, *23*, 622; (b) Eppinger, J.; Funeriu, D. P.; Miyake, M.; Denizot, L.; Miyake, J. *Angew. Chem., Int. Ed.* **2004**, *43*, 3806; (c) Huang, J.; Zhu, H.; Haggarty, S. J.; Spring, D. R.; Hwang, H.; Jin, F.; Snyder, M.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 16594.
6. (a) Roth, J. *Chem. Rev.* **2002**, *102*, 285; (b) Ritchie, G. E.; Moffatt, B. E.; Sim, R. B.; Morgan, B. P.; Dwek, R. A.; Rudd, P. M. *Chem. Rev.* **2002**, *102*, 305; (c) Bertozzi, C. R.; Kiessling, L. L. *Science* **2001**, *291*, 2357.
7. Drickamer, K.; Taylor, M. E. *Genome Biol.* **2002**, *3*, 1034.1.
8. Carbohydrate microarrays have been utilized for the rapid analysis of carbohydrate–protein interactions and the detection of pathogens: (a) Shin, I.; Park, S.; Lee, M.-r. *Chem. Eur. J.* **2005**, *11*, 2894; (b) Lee, M.-r.; Shin, I. *Org. Lett.* **2005**, *7*, 4269; (c) Lee, M.-r.; Shin, I. *Angew. Chem., Int. Ed.* **2005**, *44*, 2881; (d) Shin, I.; Cho, J. W.; Boo, D. W. *Comb. Chem. High Throughput Screening* **2004**, *7*, 565; (e) Feizi, T.; Chai, W. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 582; (f) Disney, M. D.; Seeberger, P. H. *Chem. Biol.* **2004**, *11*, 1701; (g) Nimrichter, L.; Gargir, A.; Gortler, M.; Altstock, R. T.; Shtevi, A.; Weisshaus, O.; Fire, E.; Dotan, N.; Schnaar, R. L. *Glycobiology* **2004**, *14*, 197; (h) Blixt, O.; Head, S.; Mondala, T.; Scanlan, C.; Huflejt, M. E.; Alvarez, R.; Bryan, M. C.; Fazio, F.; Calarese, D.; Stevens, J.; Razi, N.; Stevens, D. J.; Skehel, J. J.; van Die, I.; Burton, D. R.; Wilson, I. A.; Cummings, R.; Bovin, N.; Wong, C.-H.; Paulson, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17033; (i) Park, S.; Lee, M.-r.; Pyo, S.-J.; Shin, I. *J. Am. Chem. Soc.* **2004**, *126*, 4812; (j) Wang, D.; Liu, S.; Trummer, B. J.; Deng, C.; Wang, A. *Nat. Biotechnol.* **2002**, *20*, 275; (k) Park, S.; Shin, I. *Angew. Chem., Int. Ed.* **2002**, *41*, 3180; (l) Fukui, S.; Feizi, T.; Galustian, C.; Lawson, A. M.; Chai, W. *Nat. Biotechnol.* **2002**, *20*, 1011.
9. Lectin microarrays have been also developed for profiling glycans on proteins or cells: (a) Hsu, K.-L.; Pilobello, K. T.; Mahal, L. K. *Nat. Chem. Biol.* **2006**, *2*, 153; (b) Kuno, A.; Uchiyama, N.; Koseki-Kuno, S.; Ebe, Y.; Takashima, S.; Yamada, M.; Hirabayashi, J. *Nat. Methods* **2005**, *2*, 851; (c) Zheng, T.; Peelen, D.; Smith, L. M. *J. Am. Chem. Soc.* **2005**, *127*, 9982; (d) Pilobello, K. T.; Krishnamoorthy, L.; Slawek, D.; Mahal, L. K. *ChemBioChem* **2005**, *6*, 985; (e) Angeloni, S.; Ridet, J. L.; Kusy, N.; Gao, H.; Crevoisier, F.; Guinchard, S.; Kochhar, H.; Sigrist, S.; Sprenger, N. *Glycobiology* **2005**, *15*, 31.
10. Lee, M.-r.; Jung, D.-W.; Williams, D.; Shin, I. *Org. Lett.* **2005**, *7*, 5477.
11. Liener, I. E.; Sharon, N.; Goldstein, I. J. *The Lectins: Properties, Functions, and Applications in Biology and Medicine*; Academic: Orlando, 1986.
12. Gao, Y.; Eguchi, A.; Kakehi, K.; Lee, Y. C. *Bioorg. Med. Chem.* **2005**, *13*, 6151.
13. (a) Capila, I.; Linhardt, R. J. *Angew. Chem., Int. Ed.* **2002**, *41*, 390; (b) Jordan, R. E.; Kilpatrick, J.; Nelson, R. M. *Science* **1987**, *237*, 777.
14. (a) Jackson, R. L.; Busch, S. J.; Cardin, A. D. *Physiol. Rev.* **1991**, *71*, 481; (b) Kjellén, L.; Lindahl, U. *Annu. Rev. Biochem.* **1991**, *60*, 443.
15. (a) Walzel, H.; Hirabayashi, J.; Kasai, K.-i.; Brock, J.; Neels, P. *Glycoconjugate J.* **1996**, *13*, 99; (b) Dupuis, G.; Bastin, B. *J. Leukocyte Biol.* **1988**, *43*, 238.
16. (a) Ashraf, M. T.; Khan, R. H. *Med. Sci. Monit.* **1980**, *9*, RA265; (b) Hume, D. A.; Weidaman, J. *Mitogenic Lymphocyte Transformation*; Elsevier/North-Holland Biomedical: Amsterdam, 1980.
17. Allen, A. K.; Neuberger, A.; Sharon, N. *Biochem. J.* **1973**, *131*, 155.